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TECHNICAL REPORT
BWL 19

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USE OF CENTRIFUGATION TO CLARIFY WHOLE-EGG SLURRY INFECTED WITH COXIELLA BURNETII (U)

WILLIAM C. PATRICK, III
JACK L. DAVIS

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U.S. ARMY CHEMICAL CORPS
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BWL Technical Report 19

USE OF CENTRIFUGATION TO CLARIFY WHOLE-EGG SLURRY INFECTED WITH *COXIELLA BURNETII* (C)

William C. Patrick III

Jack L. Davis

Pilot Plants Division
DIRECTOR OF DEVELOPMENT

Projects 4-92-02-030
4-92-02-034

August 1959

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(U) FOREWORD

(U) This investigation was conducted under Project 4-92-02-030. Sub-project -01, "Unit Operations and Processes For BW Agents," Task 9. The expenditure order was 80-71-500.

(U) The authors are grateful to Mr. Wendell H. Kayser, Deputy Director of Development and to Mr. Lou C. Dixon, MD Division, for defining certain product characteristics that could improve dissemination efficiencies of spray-type munitions.

(C) ABSTRACT

(C) Centrifugation was investigated as a method for removing coarse particles of tissue from whole-egg slurry infected with Coxiella burnetii. These particles must be removed if the slurry is to be disseminated from spray-type munition systems such as the E120 and the North American spray nozzle. However, the removal must not reduce the concentration of rickettsiae in the supernatant liquid or product. A condition of centrifugation was determined experimentally which resulted in a product which met the requirements of the E120 munition. Centrifugation, supplemented by filtering the slurry through a series of screens, resulted in a product which met the requirements of the North American spray nozzle system.

(C) The supernatant product from the centrifugation study was stored at 4°C to determine biological decay of the organism with time. The data indicated that, under optimum conditions, there is no loss in viability for 150 days.

(U) A reference slurry was established, and incorporated in the assay program. Assessment variability still remains one of the major obstacles in defining the influence of controlled variables on rickettsial populations.

(C) Freshly prepared suspensions of slurry were aerosolized with the PT-12 nozzle. Temperature and humidity have little influence on biological decay of the organism. Concentration of organisms is reduced primarily by physical fall-out of particles.

(C) DIGEST

(C) Centrifugation was investigated as a method for removing coarse particles of tissue from whole-egg slurry infected with Coxiella burnetii. These particles must be removed if the slurry is to be disseminated from spray-type munition systems such as the E120 and the North American spray nozzle. The removal must not reduce the concentration of rickettsiae in the supernant liquid or product. A condition of centrifugation was determined experimentally which resulted in a product which met the requirements of the E120 munition. Centrifugation, supplemented by filtering the slurry through a series of screens, was required for a product which met the requirements of the North American spray nozzle system.

(C) The supernatant product from the centrifugation study was stored at 4°C to determine biological decay of the organism with time. The data indicated that, under optimum conditions, there is no loss in viability for 150 days.

(U) The procedure for estimating rickettsial concentration of the slurries was examined critically. A reference slurry was established, and incorporated in the assay program. Precision of the assay procedure was improved; however, assessment variability still remains one of the major obstacles to defining the influence of controlled variables on rickettsial populations.

(C) The aerosol characteristics of the organism were studied. Freshly prepared suspensions of slurry were aerosolized with the PT-12 nozzle. These studies demonstrated that temperature and humidity have little influence on biological decay of the organism. Concentration of organisms is reduced primarily by physical fall-out of particles from the cloud.

(C) Studies of egg slurries containing not Coxiella burnetii but a simulant, Bacillus subtilis var. niger, showed that munition efficiency increased as the thixotropic nature of the slurry was reduced.

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I. (C) INTRODUCTION

A. (C) PURPOSE

(C) The major objective of this program was to develop an egg slurry infected with Coxiella burnetii that could be disseminated from spray-type munitions. The product scheduled for improvement, a milled suspension of infected embryonated egg, contains particles which do not pass the orifice of these spray-type munitions and therefore are not effectively disseminated from them. Centrifugation was selected as a method which would remove the undesirable particulate matter from the slurry without reducing the rickettsial content of the product. This study also provided an opportunity to obtain additional information concerning (a) precise estimates of the variability of the assay procedure for Coxiella burnetii, (b) stability of slurry stored at + 4°C, (c) the biological decay rate of the organism in aerosol, (d) the feasibility of filtering whole-egg slurry, and (e) the influence on aerobiological properties of changing the physical properties of slurry by dilution.

B. (C) BACKGROUND

(C) Procedures for producing Coxiella burnetii in whole-egg slurry were developed by personnel in Virology I Branch, Virus and Rickettsia (VR) Division. These procedures were adapted and modified for use in the Pilot Plant between 1951 and 1953. An experimental operating manual was written for the Pilot Plant.1/* Data obtained during this period are summarized in Technical Memorandum Report 2-24.2/

(C) The product from this process is a slurry obtained by milling embryonated egg infected with Coxiella burnetii through a colloid mill. The biological and physical properties of the product are shown in Table I. The product met all specifications for dissemination from the M114. However, it is not suitable for use in spray-type munitions now being developed. The slurry contains a small percentage of coarse particles that do not pass the orifice of a munition such as the E120. These coarse particles are feathers, bones, and other tissues which cannot be milled to particles less than 0.06 inch in diameter. The standard Pilot Plant slurry must be clarified of this undesirable tissue in order to meet the requirements of a variety of munition devices.

* See Literature Cited.

TABLE I. (C) BIOLOGICAL AND PHYSICAL PROPERTIES OF WHOLE-EGG SLURRY PREPARED IN PILOT PLANT, 1951 TO 1953

Product Characteristic	Result
1. Rickettsial titer (Log_{10} $GPIPID_{50}$/ml)[a]	10.04
2. Rickettsial titer, 95% Confidence Limits	not established
3. Particle Size[b]	0.063 inch
4. Viscosity[c]	26 - 33 centipoises
5. Total dry solids[d]	23 per cent
6. Specific Gravity[e]	1.035
7. pH[f]	7.1 - 7.5

a. Log_{10} guinea pig intraperitoneal infective dose, 50 per cent.
b. The orifice diameter through which 64 ml of slurry will pass without plugging orifice when 30 psig is employed.
c. Viscosity - measured at 25°C with a Brookfield viscosimeter using No. 1 spindle at 50 and 30 rpm.
d. Determined by method of Flosdorff and Webster.
e. Slurry held at 25°C and gravity measured by Balling hydrometer.
f. pH measured with Beckman pH meter.

II. (C) EXPERIMENTAL PROCEDURES

A. (C) INITIAL INVESTIGATION

(C) It was decided that this study should be directed toward meeting two requirements: (a) that the whole-egg slurry product be capable of passing the orifice of the E120 munition, and (b) that the product contain at least 1×10^{10} doses per milliliter capable of infecting 50 per cent of the guinea pigs inoculated. The E120 orifice (0.010 inch) was selected because it was thought there would be little interest in smaller orifices for the spray-type devices. The requirement for rickettsial concentration was selected because it represented the average agent concentration obtained from 30 lots of infected slurry produced between 1951 and 1953.

(U) A preliminary investigation determined the degree of centrifugation required to remove coarse tissue particles from slurry prepared from normal 18-day-old embryos (Appendix A). Speeds of 30,000 revolutions per minute and flow rates of 100 to 500 milliliters per minute in the Sharples Pressurtite Centrifuge were required before the product would pass an orifice of 0.010 inch. It was believed that these conditions of centrifugation would sediment a large percentage of the rickettsiae.

(U) Subsequent investigations with infected slurry prepared from freshly made seed are reported in Appendix B.

B. (U) DEVELOPMENT OF SELECT-HARVEST PROCEDURES

(U) Since preliminary data with normal slurry indicated that centrifugation would probably remove 30 to 50 per cent of the rickettsiae of the product, it was decided that an alternate approach to the problem of clarifying the slurry by centrifugation would be to use a selective harvesting procedure. This decision was based on the fact that the embryo itself is not a rich source of rickettsiae. Moreover, since the 15-day-old embryo is characterized by well-defined feather, bone and cartilage development, it is difficult to process into an acceptable product. The composition of a 15-day-old embryonated egg is shown in Table II. A selective harvesting procedure was developed for infected eggs whereby the embryo was discarded with the shell (Appendix C). The remaining tissues and fluids were processed into a "select-harvest" product.

(U) Four lots of 2000 eggs each were inoculated with Coxiella burnetii and half the eggs were harvested by the whole-egg procedure described in the manual.[1] Time and motion studies were made for each harvest. Two

TABLE II. (U) SOME PHYSICAL CHARACTERISTICS OF COMPONENTS OF 15-DAY-OLD EMBRYONATED EGG

Egg Components	pH	Per Cent Solids	Weight, gm	Per Cent of Total Weight
1. Embryo	7.1	11.4	11.8	21.2
2. Yolk[a]/	7.5	36.5	9.3	16.7
3. Albumin	7.8	29.6	8.6	15.5
4. Allantoic Fluid	---		5.9	10.6
5. Yolk Sac[a]/	---		3.5	6.3
6. Amniotic Fluid	---		2.6	5.0
7. Extra Embryonic Membranes	---		1.2	2.0
8. Miscellaneous	---		12.6	22.7
Total			55.5	100.0

a. If the embryonated eggs were infected with *Coxiella burnetii*, 90 per cent of the rickettsiae would be contained within the yolk and yolk-sac.

lots of infected eggs were milled in the Eppenbach Q-V colloid serum mill; the other two lots were milled in the Charlotte Colloid Mill, Model No. 3. The milled suspensions of slurry were stored in glass at -50°C and were removed from storage as they were required for the centrifugation study.

(U) Major differences between the select-harvest and whole-egg products are summarized in Table III. The select-harvest material is more easily milled into a product containing fewer chunks of large tissue; however, harvesting time is 48 per cent slower and 31 per cent less slurry is recovered than with the whole-egg harvest. Theoretically, the select-harvest slurry contains 21 per cent more rickettsiae per unit volume than whole-egg slurry; however, a difference in concentration between the products could not be demonstrated. The reason for this is discussed later. Additional process information is presented in Appendix D.

C. (U) PRODUCT CLARIFICATION BY CENTRIFUGATION

(U) Frozen aliquots of infected select-harvest and whole-egg slurries were thawed as required and centrifuged in the laboratory-model, Pressurtite Sharples Centrifuge. A preliminary investigation of a wide range of centrifuge conditions was made in order to determine the optimum degree of centrifugation; that is, the condition that removed maximum quantities of coarse particles from slurry without reducing the concentration of rickettsiae significantly. In a second investigation, a narrow range of centrifuge variables was studied that caused removal of the rickettsiae from the feed slurry. Table IV contains the centrifuge variables studied in both investigations.

(U) The following procedures were used in operating the centrifuge. The centrifuge was filled with 200 milliliters of milled slurry. The bowl was brought to the desired rotational speed and slurry was fed into the bowl at the desired feed rate. Bowl rotation was checked at three-minute intervals and the feed into the centrifuge was constantly metered. Since feed rates were varied from 100 to 750 milliliters per minute, a ten-minute period of centrifuge operation was used throughout. The amounts of slurry centrifuged varied from 1000 to 7500 milliliters. Bowl temperature was kept between 5° and 10°C.

(U) The volumes of feed and supernatant liquids were measured in order to calculate a material balance for the operation; however, when the bowl rotates at 30,000 revolutions per minute in combination with a slow feed input, recovery data are not precise because the bowl flings much of the supernatant liquid past the traps designed to collect it. At slower rotational speeds, precise data can be obtained for this operation.

TABLE III. (C) COMPARISON OF PROCESS LOSSES AND PHYSICAL CHARACTERISTICS OF MILLED WHOLE-EGG SLURRY AND MILLED SELECT-HARVEST SLURRY

Physical or Biochemical Property	Infected Whole-Egg Slurry	Infected Select-Harvest Slurry
Particle Size[a]	0.068	0.022
Specific Gravity	1.03	1.03
Total Solids, per cent	25.07	30.07
Rickettsial Concentration[b]	10.30	10.20
Product Recovered Per Egg, ml	44 - 46	30 - 32
Eggs Harvested Per Man Per Hour	1500	720

a. Size of orifice through which 64 ml of slurry will pass without plugging orifice when 50 psig is employed.

b. Log_{10} guinea pig intraperitoneal infective doses per ml; no significant difference between the two concentrations.

TABLE IV. (U) VARIABLES INVESTIGATED TO DETERMINE WHAT DEGREE OF CENTRIFUGATION CAUSED SEDIMENTATION OF *COXIELLA BURNETII* FROM FEED SLURRY

Test	Type of Slurry	Centrifuge Variables Investigated rpm	Flow Rate, ml/min
Initial Study	Whole-Egg	10,000	750
		20,000	500
		30,000	100
Initial Study	Select-Harvest	10,000	500
		30,000	100
Final Study	Whole-Egg	20,000	500
		25,000	250
		30,000	100
Final Study	Select-Harvest	25,000	100
		25,000	250
		30,000	500

(U) Supernatant liquids were assayed for rickettsial content by injecting aliquots of appropriate dilutions into guinea pigs, holding the pigs for 28 days, then bleeding each pig by cardiac puncture. The blood thus obtained was processed for serum and the serum tested for the presence of complement-fixing antibodies.[1]

(U) The supernatant liquid or product was tested for particle size, viscosity, and specific gravity.[1] Because the requirement of product particle size is critical in this study, the test procedure for it is described here:

> "A Cornwall syringe is calibrated to deliver two milliliters and is equipped with a 24-inch pressurized rubber tubing with 1/8-inch opening attached to the syringe inlet tubing and a 22-inch pressurized rubber tubing with 1/8-inch opening attached to the syringe outlet valve. The end of the outlet tubing is equipped with a Leur-Lok adaptor to which a hypodermic needle is attached. The hypodermic needle has a bore of known size. The inlet tubing of the syringe, which is weighted on the tip, is placed in the test liquid where it settles to the bottom of the container. The plunger of the syringe is pushed slowly eight times. If the slurry passes the needle orifice, this procedure is replicated four times. The particle size end-point of a slurry is the smallest needle orifice tested through which 64 ml of slurry will pass without plugging the orifice. Approximately 50 psig is developed in the syringe and its assembly. Slurry is not forced through the orifice by continuing to work the plunger after it appears that the needle orifice is obstructed."

(U) The conditions of centrifugation were defined in terms of a mathematical quantity, Q/ε. Q is the volumetric rate of liquid flow through the centrifuge (cm^3/sec) and ε is the factor that relates physical dimensions of centrifuge design to the theoretical capability of the centrifuge (cm^2). This ratio is represented by the equation derived by Ambler:[3]

$$Q/\varepsilon = \frac{4.6 g}{w^2 t} \qquad \log \frac{2r_2}{r_1 r_2}$$

where: $g = 981$ cm/sec^2

$r_2 = 2.21$ cm, radius of outer surface of liquid layer

$r_1 = 1.78$ cm, radius of inner surface of liquid layer

w = angular velocity about axis of rotation in radians per second

t = time, seconds

(U) Theoretically, once these conditions have been defined, it should be possible to select the optimum condition of centrifugation. The optimum condition of centrifugation for whole-egg slurry is represented by a Q/Σ value between 1.2 and 3.8 x 10^{-6} centimeters per second (Table V). There is no loss in the rickettsial concentration of the supernatant fluid under these conditions. Moreover, the product passes an orifice 0.008 inch in diameter, which is 20 per cent smaller than the required orifice diameter of 0.010 inch. Milled whole-egg slurry contains 12.5 grams of sedimentable solids per 100 milliliters of slurry; 68 per cent of these solids are removed by this degree of centrifugation. Sixteen per cent of the total volume of whole-egg slurry is fat. Five per cent of this fat is removed during centrifugation, leaving a total fat content of 15.2 per cent. Approximately 89 per cent of the centrifuge feed is recovered as supernatant product. Similar data for select-harvest slurry are shown in Table VI. The two types of slurry closely parallel one another with respect to the optimum condition of centrifugation (Figure 1). In order to obtain a given particle size for the product, the requirement of centrifugation is somewhat less for the select-harvest slurry than for whole-egg slurry. The difference in centrifugation requirements between the slurries is not significant. On the bases of time and motion data and of material balance and physical property data, whole-egg slurry appears to be the product of choice.

D. (C) MISCELLANEOUS INVESTIGATIONS

1. (U) Assay

(U) One of the purposes of this study was to obtain estimates of the error inherent in the procedures for assessment of Coxiella burnetii. Previous studies provided data concerning day-to-day variation of the products of the plant system, but within-day variation of the assay procedures had not been established.

(U) A control or reference slurry was incorporated in the program. This was a homogenized whole-egg slurry that had been filled into plastic containers, frozen, and stored at -50°C. Because it was not possible to assay a reference sample with each unknown slurry, large lots of guinea pigs were ordered at one time and the control slurry assayed from each lot of pigs. The pigs were pooled, then randomly selected for each day of assay.

(U) Initially, the assay program was composed of the following procedures. One technician prepared duplicate series of dilutions of the test sample to obtain final dilutions of 10^{-9}, $10^{-9.5}$ and $10^{-10.5}$. For each dilution, each of three guinea pigs was injected intraperitoneally with a one-milliliter aliquot. The ID_{50} endpoints of many slurries were not bracketed by this range of dilutions and, subsequently, a duplicate series of these final dilutions was prepared: $10^{-9.5}$, $10^{-10.0}$, $10^{-10.5}$, and $10^{-11.0}$. The number of guinea pigs was increased from three to six pigs per dilution with the change in dilution range.

TABLE V. (U) RECOVERIES AND PHYSICAL PROPERTIES OF SUPERNATANT OBTAINED BY CENTRIFUGING INFECTED WHOLE-EGG SLURRY

Item	Centrifuge Conditions Defined in Terms $Q/\Sigma \times 10^{-6}$ cm/sec			
	23[a]	3.8[b]	1.2[c]	0.34[d]
Centrifuge Feed Recovered as Product, per cent	93.0	89.0	84.0	76.7
Average Rickettsiae Recovered in Product, per cent	100	100	56	39
Particle Size, inch	0.013	0.008	0.006	0.004
Viscosity (25°C)	32	30	26	23
Specific Gravity	1.03	1.03	1.03	1.03
Total Solids, per cent	25.0	25.2	24.9	25.1
pH	7.2	7.2	7.2	7.3

a. 10,000 rpm, flow rate 750 ml/min; one test
b. 20,000 rpm, flow rate 500 ml/min; two tests
c. 25,000 rpm, flow rate 250 ml/min; one test
d. 30,000 rpm, flow rate 100 ml/min; two tests

TABLE VI. (U) RECOVERIES AND PHYSICAL PROPERTIES OF SUPERNATANT OBTAINED BY CENTRIFUGING INFECTED SELECT-HARVEST SLURRY

Item	Centrifuge Conditions Defined in Terms $Q/_{\Sigma}$ x 10^{-6} cm/sec			
	15.3[a]	1.7[b]	1.2[c]	0.34[d]
Centrifuge Feed Recovered as Product, per cent	90	88	89.5	74.3
Average Rickettsiae Recovered In Product, per cent	100	100	86	6
Particle Size, inch	0.008	0.006	0.004	0.004
Viscosity (25°C)	17	18	[e]	-
Specific Gravity	1.03	1.03	1.03	-
pH	7.5	7.5	7.4	7.5

a. 10,000 rpm, flow rate of 500 ml/min; one test
b. 30,000 rpm, flow rate of 500 ml/min; one test
c. 25,000 rpm, flow rate of 250 ml/min; two tests
d. 30,000 rpm, flow rate of 100 ml/min; one test
e. No data.

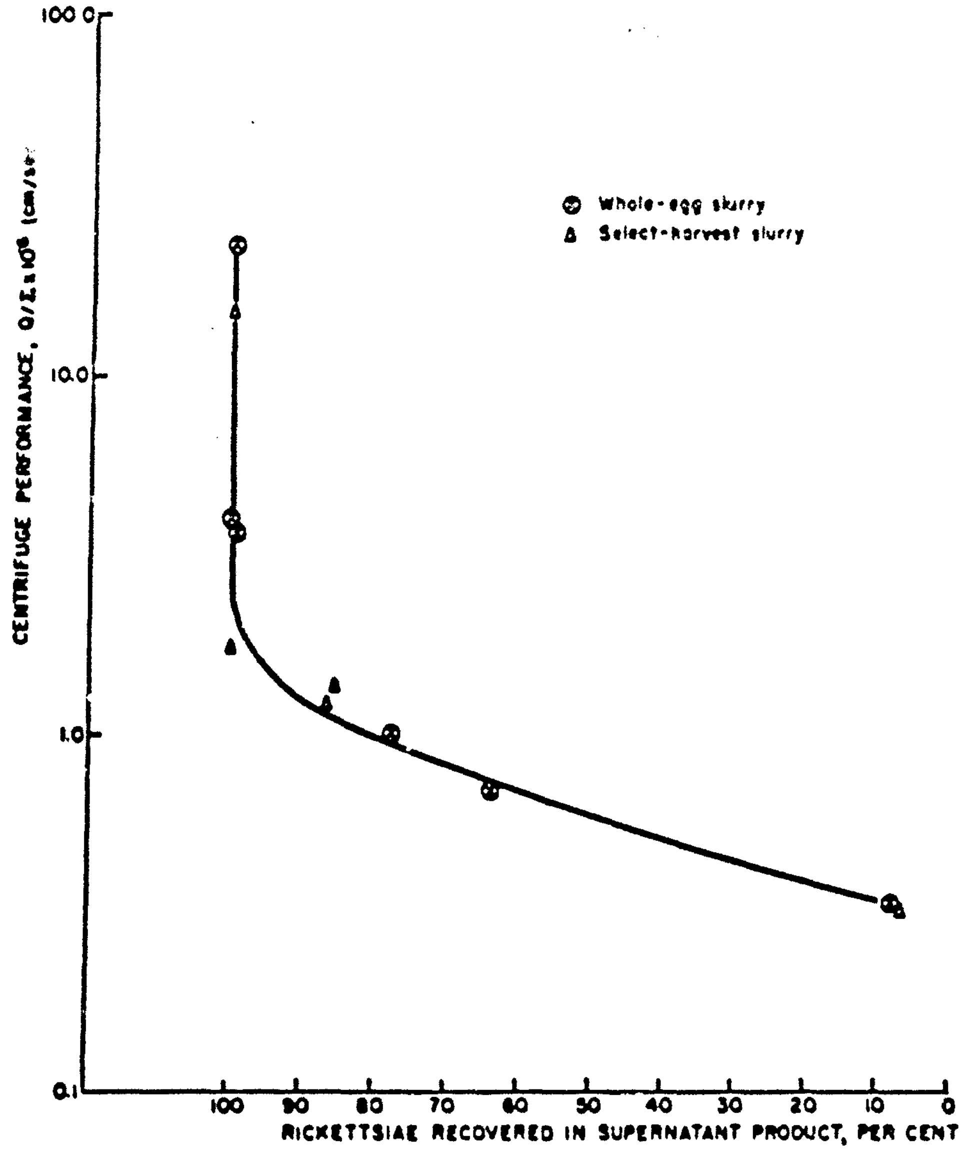

FIGURE 1. (U) INFLUENCE OF DEGREE OF CENTRIFUGATION ON AMOUNT OF RICKETTSIAE RECOVERED IN SUPERNATANT.

(U) An analysis of the data indicated that no correlation existed between reference and unknown slurries. Within-day and between-day assay variabilities are shown in Table VII. Unknown slurries are not more variable than the reference. It appears that some variable significantly influences the level of agent concentration from day to day. This influence is probably caused by the test animal although this assumption has yet to be proved. Since within-day assay variability is approximately 0.070, the most precision that could be expected from this assay procedure, at the 95 per cent level of confidence, is ± 0.55 log. The probit slope of the animal response is about 1.4, which can be used in estimating the number of test animals required to obtain a given level of assay precision. The statistical analyses of these data are summarized in Appendix E.

TABLE VII. (U) VARIABILITY OF ASSAY PROCEDURE FOR ESTIMATING RICKETTSIAL POPULATION OF REFERENCE AND UNKNOWN SLURRIES

	Within-Day Assay Variation	Between-Day Assay Variation
Reference Slurry	0.067	0.498
Unknown Slurry	0.076	0.411

(U) This study demonstrates that the reference slurry can vary as much as 2.5 logs between days. It is believed that an effective assay procedure would incorporate the preparation of one dilution series of $10^{-9.2}$, $10^{-9.9}$, $10^{-10.6}$, and 10^{-11}, and the inoculation of six guinea pigs for each dilution.

2. (C) Storage Tests at 4°C

(U) Three lots of undiluted whole-egg slurry that had been clarified by centrifugation were stored at 4°C in filled and sealed glass bottles and in half-filled glass bottles sealed with cotton. At 30-day intervals, the slurry was assayed for biological and physical properties. Properties of the slurry that had been stored to exclude air are summarized in Figure 2, those of slurry stored in the presence of air in Figure 3. Each curve represents the averaged data from three different lots of material.

(C) Product titer does not change for 150 days under either test condition. Only the titer of slurry stored in the absence of air remains stable for more than 180 days. Growth of bacterial contaminants is retarded when slurry is stored in the absence of air and therefore pH remains at a favorable level (alkaline) for the rickettsiae for at least 150 days.

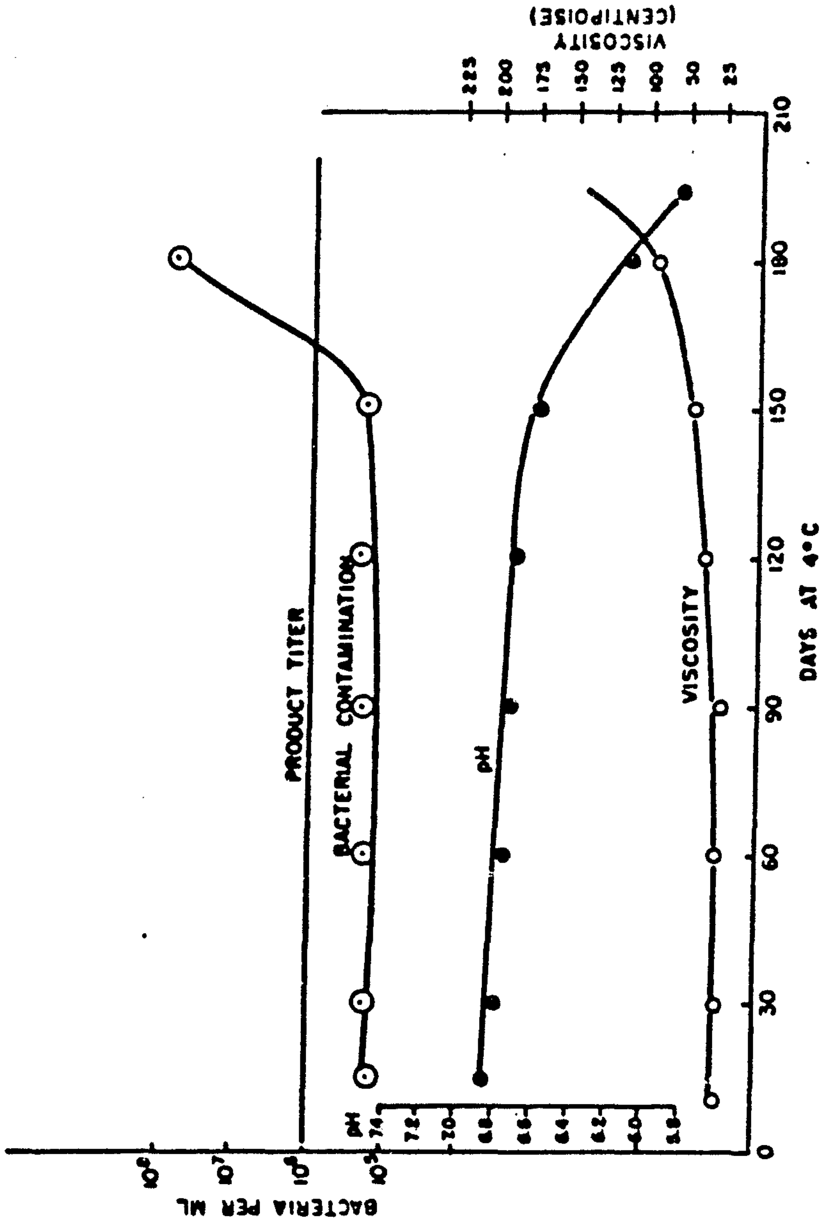

Figure 2. (C) Biological and Physical Properties of Whole-Egg Slurry Contained in Glass Bottles to Exclude Air During Storage at 4°C.

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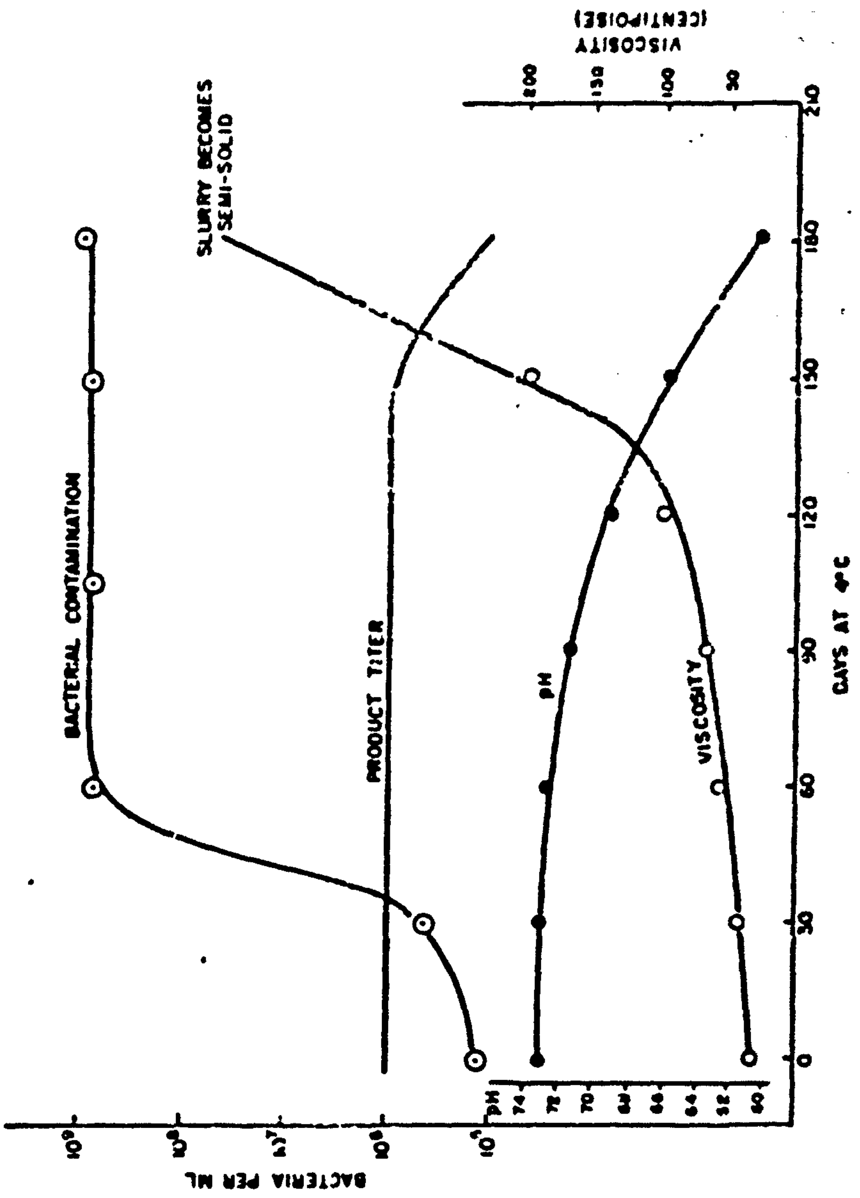

FIGURE 3. (C) BIOLOGICAL AND PHYSICAL PROPERTIES OF WHOLE-EGG SLURRY CONTAINED IN GLASS BOTTLES IN A MANNER THAT PROMOTED OXIDATION OF SLURRY STORED AT 4°C.

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(U) Since pH did not decrease, viscosity remained stable for 150 days. The presence of air promotes growth of bacteria in the slurry and the production of acid which causes the slurry to become semi-solid by the end of 180 days.

(U) There are two pertinent conclusions that can be drawn from these data. Whole-egg slurry is an excellent buffer which is able to absorb much of the acid produced by bacterial growth before pH is reduced to a level unfavorable for the rickettsiae. Secondly, storage in the absence of air is desirable.

3. (C) Influence of Physical Properties of Whole-Egg Slurry on Aerosol Recovery

(C) Three types of explosive munitions that contained whole-egg slurry infected with Coxiella burnetii were evaluated.* The slurry was a Pilot Plant product that had been clarified by centrifugation. The source strength or initial recovery from these munition tests averaged less than one per cent. It had been anticipated that this organism, because of its excellent stability, would give better recoveries than these, perhaps in the range of three to five per cent.

(U) An experiment was conducted to determine if low recoveries of this organism were a result of physical properties of the slurry. Bacillus subtilis var. niger was added to normal whole-egg slurry. Portions of the slurry were then diluted with distilled water to obtain the concentrations shown in Table VIII. The influence of diluent on the viscosity of normal slurry is shown in Figure 4. Ninety-ml aliquots of undiluted and diluted slurry were filled in the 4.5-inch explosive spheres to obtain three mass ratios (ratio between slurry and explosive). The munitions were exploded and the resulting aerosols were sampled four minutes after establishment of a stable cloud. The preliminary results of this experiment are also shown in Table VIII. Final test results were reported by Technical Evaluation Division in Report of Test 58-TE-1026.

(U) The following conclusions can be drawn: (a) the physical characteristics of the slurry prevent the efficient dissemination of B. subtilis in aerosol; (b) as the slurry is diluted, the recovery of B. subtilis increases; and (c) an optimum dilution of slurry is obtained between five parts slurry plus one part diluent and one part slurry plus one part diluent. The optimum dilution may be defined as that combination of slurry and water which maximizes the quantity of agent aerosolized.

* MD Division, Fort Detrick

TABLE VIII. (C) INFLUENCE OF DILUTING NORMAL WHOLE-EGG SLURRY CONTAINING B. SUBTILIS WITH WATER ON RECOVERY OF THE ORGANISM WHEN DISSEMINATED FROM 4.5-INCH EXPLOSIVE SPHERE

Treatment of Slurry	Viscosity, centipoise	B. subtilis Recovery, per cent d/	Efficiency, per cent Mass Ratio		
			1 a/	2 b/	3 c/
Normal egg (no dilution)	93	1.04	1.04	0.30	1.54
8 parts egg + 1 part water	10.4	1.82	1.51	No data	No data
1 part egg + 1 part water	6.2	2.88	1.44	No data	No data
1 part egg + 9 parts water	3.1	5.93	0.59	No data	No data

a. Mass ratio of 2.25 parts slurry to 1 part explosive.
b. Mass ratio of 1.00 part slurry to 1 part explosive.
c. Mass ratio of 4.40 parts slurry to 1 part explosive.
d. Recovery at four minutes after establishment of aerosol.

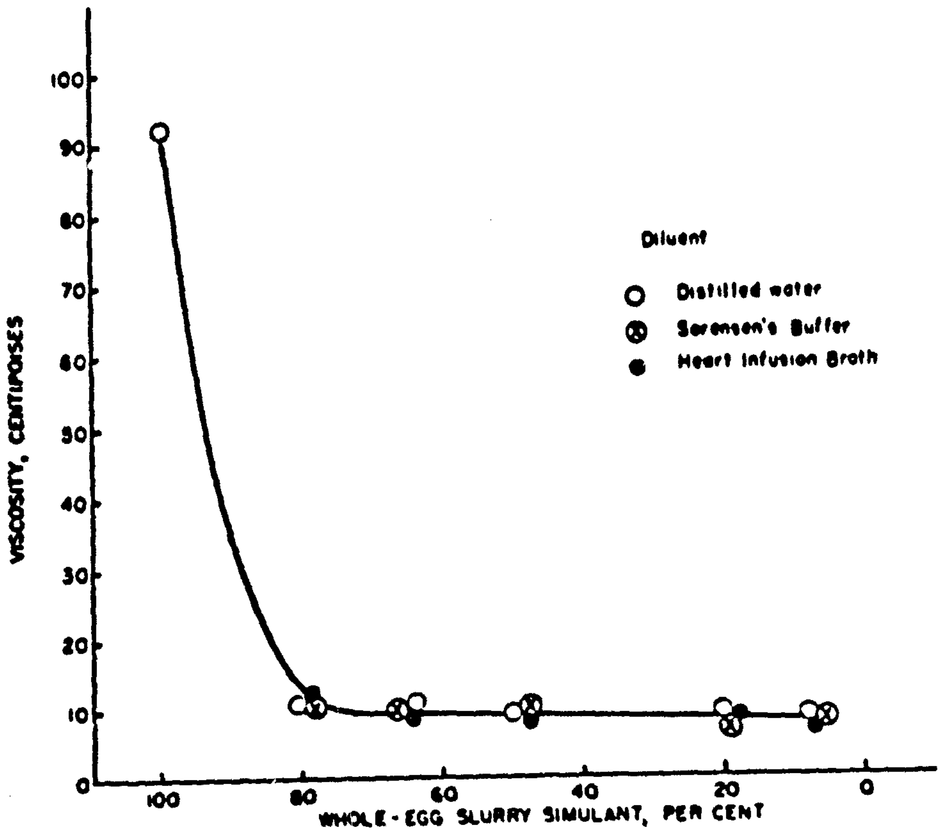

Figure 4. (U) Influence of Diluting Whole-Egg Slurry Simulant with Various Diluents to Improve Physical Properties of the Mixture (As Reflected by Viscosity).

(U) A second experiment was designed whereby the efficiency of three disseminators were tested. Each disseminator was filled with: (a) normal whole-egg slurry simulating the infected slurry; (b) normal whole-egg slurry diluted in the ratio of five parts slurry to one part distilled water; (c) the sediment obtained by centrifuging normal slurry at 35,000 revolutions per minute, feed rate 100 milliliters per minute, and the sediment diluted in the ratio of one part sediment, five parts distilled water; and (d) the water layer obtained by treating normal slurry with Freon-heptane. The disseminators were a PT-12 nozzle, an ADL nozzle, and a conical explosive device (mass ratio of three and six grams PETN to 18 milliliters of fill). All fills contained B. subtilis tracer at a concentration of 3 x 10^9 spores per milliliter.

(C) Preliminary results from this test (Tech Evaluation Division Test 58-TE-1079) are summarized in Table IX. This information confirms and amplifies data obtained in the preceding investigation; namely, that the thixotropic nature of whole-egg slurry is one of the primary factors responsible for the low recoveries of Coxiella burnetii when it is aerosolized from egg products. As viscosity and per cent solids of the product decrease, the recovery of the organism increases. This experiment provides ample justification for diluting infected slurries with distilled water in order to reduce the thixotropic nature of the slurry.

4. (C) Aerosol Assessment of Clarified Product

(C) The major objective of this portion of the study was to obtain an estimate of agent disseminated from the PT-12 nozzle and of the biological decay of the agent under various conditions of temperature and relative humidity (RH).

(C) Whole-egg slurry that had been clarified by centrifugation was tested for its aerosol properties by Technical Evaluation Division. Slurry for these tests was diluted in the ratio of two parts slurry to one part distilled water, then homogenized in the Eppenbach Mill to reduce the thixotropic nature of the product. The test slurry was stored in the absence of air in glass bottles at 4°C for no more than three weeks before it was aerosolized by the PT-12 nozzle. The aerosol was tested at the following chamber conditions: 75°F and 85 per cent RH, 75°F and 30 per cent RH, 40°F and 85 per cent RH. Each condition was tested three times. Test procedures and chamber conditions are described in detail in Appendix F and in Technical Evaluation Division Report 58-TE-1010.

(C) The contents of the impinger samplers were pooled by personnel of Technical Evaluation Division and assayed by personnel in the control laboratory of the Egg Process Section. Preliminary results from these tests are summarized in Table X. It is concluded from these results that:

TABLE IX. (U) SOURCE STRENGTH OF B. SUBTILIS TRACER RECOVERED FROM FOUR DIFFERENT EGG PRODUCTS AEROSOLIZED BY THREE DISSEMINATORS

	Source Strength of B. subtilis from Products, %			
Disseminator	A[a]	B[b]	C[c]	D[d]
PT-12 Nozzle	1.27	2.07	6.61	11.90
ADL Nozzle	12.40	13.20	28.70	36.70
Conical Explosive Device	3.78	4.62	9.20	13.20

a. Non-diluted whole-egg slurry; viscosity 32 centipoise; total solids 23 per cent.
b. Five parts whole-egg slurry plus 1 part distilled water; viscosity 17 centipoise; total solids 21.2 per cent.
c. Sediment from high-speed centrifugation diluted in ratio of 1 part sediment, 5 parts distilled water; viscosity 6 centipoise; total solids 5.6 per cent.
d. Water layer from Freon-heptane extraction of normal slurry; viscosity 3.8 centipoise; total solids 1.6 per cent.

TABLE X. (C) SUMMARY OF ASSAY DATA OBTAINED BY INOCULATING IMPINGER FLUIDS INTO GUINEA PIGS

Impinger Samplers Were Exposed to Aerosol at Various Intervals Following Creation of the Cloud

Test Conditions RH, %	Temp, °F	Control Slurry, $GPIPID_{50}$/ml	Guinea Pig ID_{50}/ml[a] of Impinger Fluid Obtained From Aerosol Cloud at Following Time Intervals 4 Min.	93 Min.	182 Min.	271 Min.	360 Min.
85	75	11.47	5.00	4.86	3.71	3.50	2.60
		11.08	3.74	3.09	3.61	2.67	2.67
		9.83	4.00	3.84	2.81	2.75	2.50
35	40	11.20	5.36	5.16	4.38	2.85	2.64
		10.90	3.60	3.52	2.91	3.44	2.60
		12.00	4.34	4.43	3.57	3.10	2.16
30	75	11.88	3.89	2.86	2.45	3.09	1.61
		11.37	3.66	3.62	2.55	1.71	1.90
		10.48	3.10	2.85	2.61	2.08	2.24

a. All assays reported log to the $base_{10}$.

(a) There is practically no biological decay of organisms during the six hours of testing. The average total decay for all test conditions was 1.11 per cent per minute of aerosol age.

(b) Source strengths from the PT-12 nozzle ranged from 0.008 to 1.0 per cent. This recovery is lower than anticipated but it confirms similar data obtained with Pilot Plant product aerosolized in a series of tests co-sponsored by MD and TK Divisions.

(c) There were no significant differences in decay rates among the three test conditions.

(d) A respiratory ID_{50} endpoint for exposed guinea pigs was not obtained because all test animals became infected. An average of 417 $GPIPID_{50}$ doses remained in the chamber at the end of six hours. This is approximately 32 times more rickettsiae than are required to produce one infectious respiratory dose (guinea pig).

5. (C) Filtration of Whole-Egg Slurry

(U) During the centrifugation study, the Egg Process Section was required to produce a whole-egg simulant product that would pass an orifice of 0.006-inch diameter. This slurry was required by the Directorate of Development for a contract with North American Aviation Corporation (Contract DA-18-064-404-CML-338). Several gallons of simulant were produced which, when tested by the procedure for determining particle size as described previously, passed the required orifice. Three lots of slurry were shipped to North American Aviation Corp. and all three lots failed to disseminate properly. The contractor filtered portions of the slurry through gauze and Whatman Filter paper No. 41. The debris that plugged the test orifices was photographed and found to be particles with a density of one or less (Figure 8). These particles were not removed by centrifugation nor were they detected by the particle-size test procedure. Much of this debris floats in whole-egg slurry, which has a specific gravity of 1.036 at 25°C. It was also noted for the first time that our procedure for checking particle size tests only the liquid at the bottom of the slurry container.

(U) This information demonstrated the need of a screening operation for removing coarse particles of light density. A series of fine screens was installed in the plant system. Two stainless steel screens of 100 mesh were installed to filter the slurry after centrifugation. Three screens of 120 mesh were installed in the filling hood to filter slurry immediately after it leaves the plant system and just previous to filling. These screens provided a basic means for filtering 120 gallons of whole-egg slurry simulant and 117 gallons of whole-egg slurry infected with Coxiella burnetii. One basic modification was made to this screening system. During the course of filtering the quantities of slurry described above, it was

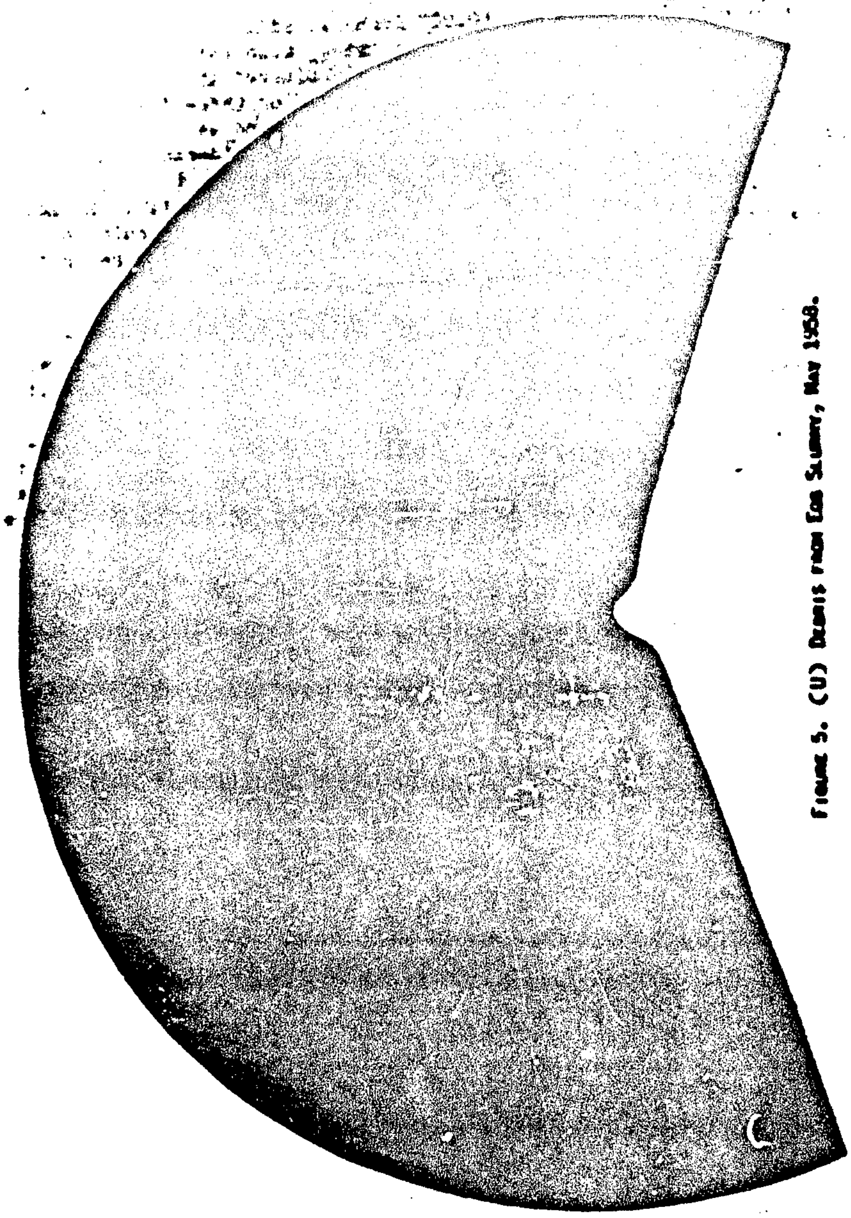

FIGURE 5. (U) DEBRIS FROM EOB SLURRY, MAY 1958.

observed that the 120-mesh screens (0.008 inch) in addition to retaining discrete particles of feathers, hair, bones, etc., also collected slime-like material (probably fat). The slime quickly plugged these orifices and made it necessary after filtering approximately two or three liters to stop the operation, pull the screens from their canister, and wash them. Six layers of gauze superimposed on the screens removed considerable quantities of fat. Fresh layers of gauze were installed following the filtering of each four liters of slurry because of the quantity of fat removed and not because of a reduction of flow through the filter. The gauze could be changed quickly and did not slow the operation. Later in the investigation, standard milk filters replaced the gauze.

(C) During the spraying of normal whole-egg slurry simulant from the single-fluid nozzle system developed under the North Amer an contract, it was observed that slurry that had been filtered through Wh. tman's Filter Paper No. 41 had a nozzle efficiency of approximately 18 to 20 per cent. Slurry that had not been passed through the filter paper had a nozzle efficiency of approximately 8 to 11 per cent. Although the fil. red product had physical properties like those of non-filtered slurry, some slime-like material must have been removed by the paper; however, only 200 milliliters of normal slurry could be filtered through this paper (size 18 cm) before the filter paper became plugged.

(U) It can be concluded from these data that an improved slurry product can be achieved through the judicious addition of water which reduces the thixotropic nature of the product.

III. (C) CONCLUSIONS

(C) The major aim of this study was to investigate centrifugation as a method for removing particulate matter from whole-egg slurry. This particulate matter prevented whole-egg slurry infected with Coxiella burnetii from being efficiently disseminated from spray-type munitions now under development. It was essential, then, to select experimentally a condition of centrifugation which removed the undesirable particles from the slurry without reducing the rickettsial concentration of the product. In initial studies, an estimate of the centrifugal force required to remove these particles was obtained with non-infected whole-egg slurry that simulated the infected product. However, it was found that the required centrifugal force also removed 30 to 50 per cent of the rickettsiae. A select-harvest technique was therefore developed to augment the centrifugation. In this method of harvest, the embryo, which is the major source of the coarse particles, is discarded with the egg shell as waste. On the basis of time and motion studies, material balance data, and biological and physical properties of the product obtained from select-harvest and whole-egg harvest, the product of choice is the whole-egg slurry. The whole-egg technique of harvesting is faster, more product is obtained from each egg, and the product contains less solids. There was no measurable difference in rickettsial titer between the two products.

(U) These studies demonstrate that it is not possible by milling alone to produce either a whole-egg or a select-harvest slurry that meets the requirement that the slurry pass an orifice 0.010 inch in diameter. It is possible to select a condition of centrifugation for either slurry that removes all of the coarse particles that plug an 0.010-inch orifice without reducing the rickettsial content of the supernatant product. The conditions of centrifugation that achieve these product requirements are represented by a Q/Σ between 1.2 and 3.8 x 10^{-5} centimeters per second. This degree of centrifugation, which removes 68 per cent of the total sedimentable solids in whole-egg slurry, can be scaled up to the commercially available Sharples No. 16 production centrifuge.

(U) The establishment of a quality control program for the assay demonstrated that rickettsial titer of a reference slurry could vary as much as 2.5 logs between days. There was no significant difference between assay variability of unknown slurries produced in the pilot plant and that of the reference slurry. In both cases within-day sample variance was 0.070 when six test animals were inoculated per dilution. This degree of variability means that the maximum precision that could be expected from the assay procedure is ± 0.55 log. The theoretical variability predicted from the slope of guinea pig response (r = 1.4) indicates that the assay procedure is achieving the precision possible with the numbers of test animals used.

(U) These studies indicate that the following assay procedure will give the maximum precision with acceptable economy: (a) one technician prepares a single series of dilutions of $10^{-9.2}$, $10^{-9.9}$, $10^{-10.6}$, $10^{-11.3}$, and $10^{-11.9}$ for both an unknown slurry and a reference slurry; (b) five groups of six guinea pigs each are used (one group for each dilution) and each pig is inoculated with one ml of the pertinent dilution; (c) the unknown and reference slurries are assayed as described in (a) and (b) on multiple days using different lots of test animals. The major obstacle in accurately estimating the rickettsial content of a particular slurry is probably the extreme variability in response of guinea pigs from day to day. Between-day variance, even for the reference slurry, is 0.498. This means that normal variation for a control slurry is ± 1.40 logs at the 95 per cent level of confidence. On the basis of this variation, the reference slurry ostensibly can gain or lose 96.0 per cent rickettsial titer on any given day. Multiple days of assay reduce the magnitude of variation and should produce a more accurate estimate of the rickettsial population.

(C) Whole-egg slurry, when stored in glass in the absence of air at 4°C, does not deteriorate biologically nor physically for 180 days. After storage for 150 days at 4°C, slurry deteriorates if stored under similar conditions except that air is not excluded. Under this latter condition, approximately one log of titer is lost between 150 and 180 days; moreover, product ultimately becomes semi-solid because of an acid pH.

(C) The thixotropic nature of whole-egg slurry is one of the important factors responsible for the low recoveries of organisms when the slurry is aerosolized. Studies made with whole-egg simulant containing B. _subtilis_ indicate that better recoveries are obtained from products that have been derived from whole-egg slurry by purification procedures.

(C) Aerosol data obtained by disseminating centrifuged, diluted whole-egg slurry from the PT-12 nozzle indicate that: (a) a rickettsial recovery of less than one per cent is obtained initially; (b) there is practically no biological decay at test conditions of 75°F and 85 per cent RH, 75°F and 30 per cent RH, and 40°F and 85 per cent RH (total decay for these conditions averaged 1.11 per cent per minute of aerosol age); and (c) the guinea pig respiratory ID_{50} is less than 417 guinea pig intraperitoneal ID_{50}'s. This was the smallest quantity of agent remaining in the test chamber at the end of six hours of sampling.

(U) Filtering whole-egg slurry through a series of stainless steel screens removes coarse particles of low specific density that are not removed by centrifugation. The incorporation of either several layers of gauze or a milk filter in the filtration operation eliminates frequent washing of the screens and produces a more refined product.

(U) Centrifuging whole-egg slurry results in a product that passes an orifice 0.010 inch in diameter. Centrifugation, supplemented by filtration through a series of stainless steel screens, results in a product that will pass an orifice of 0.008 inch in diameter. Centrifugation, supplemented by a series of screens and a standard milk filter, results in a product that will pass an orifice of 0.005 inch in diameter.

(C) An idealized process for infected whole-egg slurry in the Pilot Plant is presented in Figure 6. Characteristics of the product obtained from this process are compared with those of the product obtained by procedures given in the EOP, Table XI.[1] The final product should be a homogenate consisting of two parts slurry and one part water. The thixotropic properties of the slurry are reduced to such an extent that a gain in munition efficiency is obtained which more than compensates for the resulting loss in agent concentration.

(C) It is concluded that it is possible to produce a whole-egg slurry by centrifugation and filtration procedures that will meet both agent-concentration and particle-size requirements. The amount of filtration must increase as the size of the orifice of the disseminating device is reduced.

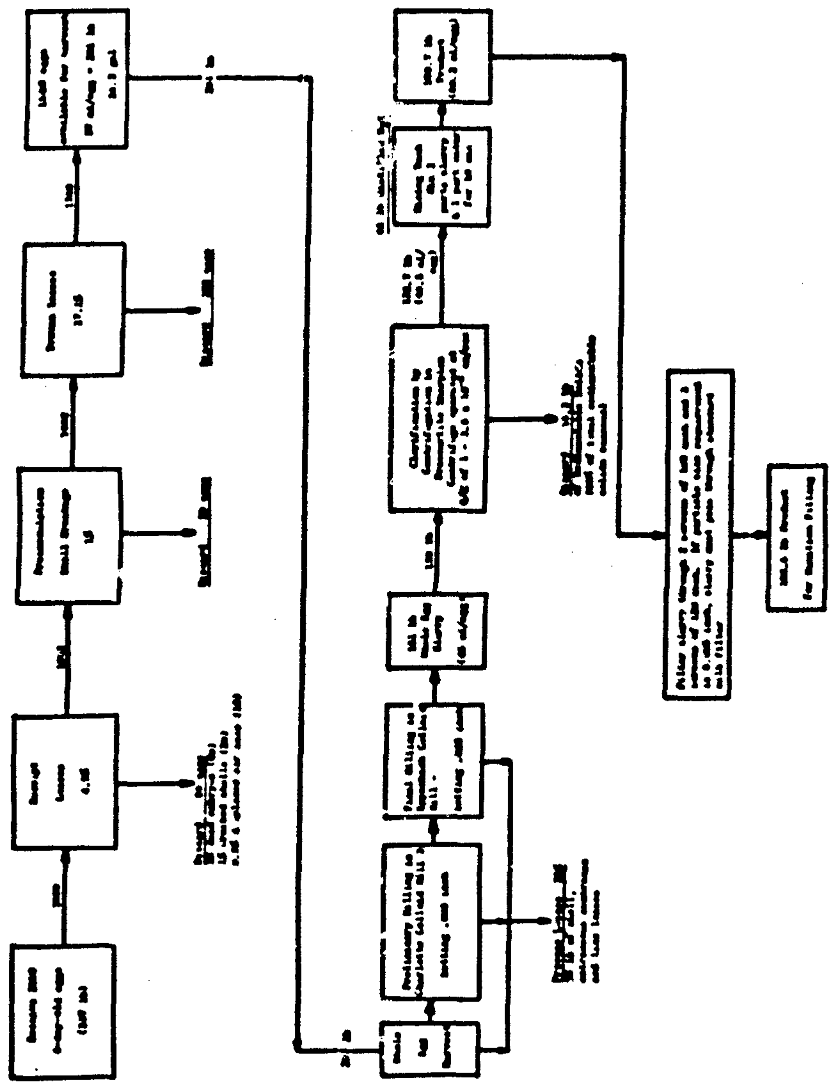

FIGURE 6. (C) FLOW DIAGRAM OF AN IDEAL PILOT PLANT PROCESS FOR PRODUCING A WHOLE-EGG SLURRY CONTAINING COXIELLA BURNETII. (C)

TABLE XI. (C) COMPARISON OF TYPICAL BIOLOGICAL AND PHYSICAL PROPERTIES OF CLARIFIED[a] AND NON-CLARIFIED[b] WHOLE-EGG SLURRY PRODUCED IN PILOT PLANT

Product Characteristic	Clarified Product	Non-Clarified Product
1. Rickettsial titer (Log_{10} $GPIPID_{50}$ per ml)	10.40	10.04
2. Rickettsial titer 95% confidence	± 0.55 logs	Not adequately established
3. Particle Size[c]	0.005 inch	0.063 inch
4. Viscosity (centipoise) at 25°C	8 - 10	26 - 33
5. Per cent total dry solids	16.6	25
6. Specific gravity	1.025	1.035
7. pH	7.1 - 7.5	7.1 - 7.5
8. Bacterial contamination	Not Controlled	Not Controlled
9. Per cent sedimentable solids removed by centrifugation	65	None (12.1 gms solids per 100 gms slurry)
10. Per cent fat content (by volume)	9.5 - 10.5	10 - 12.5

a. Clarified product obtained from procedures developed during 1958; namely whole-egg slurry is milled, centrifuged, diluted two to one with distilled water and screened or filtered.
b. Non-clarified product obtained from procedures developed during 1951-1953. The slurry is milled only.
c. The orifice diameter through which 64 ml of slurry will pass without plugging orifice when 50 psig is employed. The clarified product has been tested more thoroughly because 8 liters of slurry have passed the stated orifice using 32 psig.

LITERATURE CITED

1. Technical Manual, "Experimental Operating Procedures for Studies with Coxiella burnetii in the Virus Pilot Plant (C)," Pilot Plants Division, Fort Detrick, Frederick, Maryland, October 1953. SECRET (56-CD-4842).

2. Technical Memorandum 2-24, "Summary of Studies of Coxiella burnetii Made in the Pilot Plant During 1951-53 (C)," Fort Detrick, Frederick, Maryland, July 1958. SECRET (58-FDS-994).

3. Ambler, C.M.: "The Evaluation of Centrifuge Performance," Chem. Eng. Progress 48:150-158, 1952.

APPENDIXES

APPENDIX A

(U) CENTRIFUGATION REQUIREMENTS FOR UNINFECTED WHOLE-EGG SLURRY

(U) The major aim of this experiment was to determine the degree of centrifugation necessary to remove particles of tissue from normal whole-egg slurry so that it could pass an orifice 0.010 inch in diameter. Normal 15-day-old embryonated eggs (not infected with Coxiella burnetii) were harvested. The contents of each egg, except the shell, were homogenized in an Eppenbach Colloid Mill set with 0.012-inch clearance between rotor and stator. The slurry was stored in glass bottles at -50°C. Aliquots were thawed and centrifuged in the Sharples Laboratory Model, Pressurtite Centrifuge under the conditions indicated in Table I. A material balance was calculated for each condition of centrifugation in order to determine the amount of sedimentable solids removed from the feed slurry. The product was tested for particle size by the procedure described in the Experimental Operating Procedure.1/*

(U) Data obtained from the experiment are summarized in Table I. The minimum degree of centrifugation that achieved the desired particle size was a flow rate of 100 milliliters per minute in combination with a bowl speed of 20,000 revolutions per minute. Two other conditions of centrifugation also resulted in a product meeting particle-size requirements; however, both were at bowl speeds of 30,000 revolutions per minute which would remove from the feed a large percentage of Coxiella burnetii. This information did provide an estimate of the conditions of centrifugation that would most likely result in a product meeting particle-size requirements.

* See Literature Cited.

TABLE I. (U) PARTICLE SIZE AND RECOVERY DATA OBTAINED BY CENTRIFUGING NORMAL, MILLED WHOLE-EGG SLURRY AT VARIOUS CONDITIONS IN SHARPLES CENTRIFUGE

Feed Rate of Slurry, ml/min	10,000 rpm		20,000 rpm		30,000 rpm	
	Particle Size[a]	Feed Recovered as Product, %	Particle Size[a]	Feed Recovered as Product, %	Particle Size[a]	Feed Recovered as Product, %
1000	0.016	97.6	0.016	96.0	0.020	93.0
500	0.013	97.0	0.013	95.0	0.010[b]	93.0
100	0.016	94.0	0.010[b]	92.4	0.010[b]	89.0

a. Diameter of orifice through which slurry can be passed under differential pressure of 50 psig. Slurry, before centrifugation, passed an orifice of 0.071 inch.

b. Product meets particle-size requirements; will pass through the orifice of the E120 munition.

APPENDIX B

(U) SEED PROGRAM

(U) When the current program was established, the Pilot Plant had supplies of stock seed (SS) and plant seed (PS) on hand. These seeds had been produced in 1952 but had never contained the concentration of rickettsiae considered normal. The seeds had been prepared from embryonated eggs produced during a period when it was thought that the laying mashes contained antibiotics. It was decided to discard all previous seeds and use fresh seed supplies for the 1957-1958 program.

(U) An ampoule of certified seed (AD-1y) was obtained from Virology I Branch, VR Division, on 1 August 1957. Procedures for preparing the fresh seed stock ($2SS_1$-Q) were identical to those specified by PP Division.1/* A 1:10 dilution of certified seed produced the death pattern shown below.

Days Following Inoculation	1	2	3	4	5	6	7	8	9	10	11
Embryos Dead, %		-	16	0	0	0	2	4	22	50	12

Twenty-five eggs were selected for processing from those that died on the ninth and tenth days following inoculation. Sixty-five grams of yolk sac and yolk were harvested and processed into seed. Approximately 10.3 grams of yolk were contaminated and discarded. The remaining yolk-sac material was processed into seed and filled into 45 ampoules (4 ml/ampoule). The seed stock was not contaminated and contained $10^{5.32}$ egg doses per milliliter.

(U) On 12 September 1957 an ampoule of seed (2SS-Q) was thawed, diluted 1:1000 in broth, and inoculated into 60 embryonated eggs to produce a seed for plant use.

Days Following Inoculation	1	2	3	4	5	6	7	8	9	10	11
Embryos Dead, %	-	-	12	0	0	0	0	2	2	47	42

* See Literature Cited.

Forty eggs were selected for seed processing from those that died on the tenth and eleventh days following inoculation. Eight pools of yolk-sac material were produced and tested for sterility. None of the material was contaminated and 720 milliliters of seed were obtained. The seed was filled into ampoules, shell-frozen in a dry-ice alcohol bath and stored at -70°C. The seed contained $10^{5.85}$ egg doses per milliliter and the final sterility test was negative.

(U) A 1:1000 dilution of plant seed ($2SP_1$-Q) in broth is used in the inoculation of embryonated eggs for Pilot Plant production. This dilution should contain sufficient rickettsiae to kill 50 per cent of the embryos inoculated by the tenth day following inoculation. The death pattern of embryos inoculated with this seed demonstrates that it is highly virulent.

Days Following Inoculation	1	2	3	4	5	6	7	8	9	10
Embryos Dead, %	-	-	15	0	0	0	2	3	24	52

APPENDIX C

(U) DEVELOPMENT OF A SELECT-HARVEST PROCEDURE FOR HARVESTING EMBRYONATED EGGS INFECTED WITH COXIELLA BURNETII

(U) A technique had been developed for removing embryos infected with Venezuelan equine encephalomyelitis virus (VEE) from the other egg contents and shell. With experience, the plant operators became very proficient. It was assumed that basic elements of this procedure could be modified and used for selectively harvesting embryonated eggs infected with *Coxiella burnetii*. This organism grows to its greatest concentration in the yolk sac and yolk. Only two per cent of the rickettsiae are located in the embryo. It was assumed that if the harvesting procedure for VEE could be reversed (the embryo discarded with the shell), the remaining materials could be processed readily into a product that would pass through a 0.010-inch orifice. Previous information had demonstrated that the embryo, which at the time of harvest contained well-developed bone, cartilage, and feathers, was the component of the egg largely responsible for coarse particulate matter in whole-egg slurry.

(U) In a preliminary investigation, normal 15-day-old embryonated eggs were selectively harvested. The egg was cracked open on the air-sac end, the embryo was discarded, the remaining egg contents were removed for product by shaking them out of the shell, and the shell was discarded. A time and motion study indicated that no more than six eggs could be harvested per minute. Fifty per cent of the harvesting time was consumed in shaking the egg contents out of the shell. Subsequent laboratory investigation showed that it was possible to remove the egg contents from the shell by vacuum. The plant system was modified to permit this latter procedure to be studied thoroughly on a pilot scale. With this system, the plant operators are able to harvest between 10 and 12 eggs per minute. The harvesting procedure was separated into three components of operation in order to obtain time and motion studies. It was concluded from this study that there is little chance of significantly increasing the speed of this procedure or approaching that of a whole-egg harvest, in which 30 eggs are harvested per minute.

APPENDIX D

(U) PROCESS DATA OBTAINED FROM INFECTED SLURRY PRODUCED FOR CENTRIFUGATION STUDIES

A. (U) TRAUMATIC EGG LOSSES

(U) Embryonated eggs were inoculated in the new inoculating cabinet (Unit A) fabricated under Contract CD-6-404-137. Trays of eggs were conveyed past each inoculating station at a chain speed of 20 inches per minute. Eggs were candled for traumatic losses three days following inoculation. These data demonstrate that it is possible to inoculate eggs on a moving conveyor without excessive trauma. Traumatic losses averaged 17.3 per cent during the 1951-1953 study compared with 17.0 per cent during the current investigation.

B. (U) DEATH PATTERN OF INFECTED EGGS

(U) Following inoculation, eggs were incubated for ten days at the appropriate temperature and humidity. Death of the embryos from infection usually starts on the eighth day following inoculation. An averaged pattern of embryonic death (all egg lots) is shown in Figure 1. Eighty-one per cent of the embryos were dead at the time of harvest (ten days). This mortality rate indicates that the potency and virulence of the plant seed are quite high.

C. (U) RECOVERY DATA

(U) The select-harvest procedure recovered 32 milliliters of product per egg; 45 milliliters of product were recovered from the whole-egg harvest. These recoveries are based on the milled, non-centrifuged product.

D. (U) EQUIPMENT

(U) The present system consists of equipment adequate for the production of whole-egg slurry. The operability of the system could be improved if the two mills now in the system (Eppenbach and Charlotte) were connected in series; the Charlotte Mill should be used to homogenize the egg components into a crude slurry, which is then passed through the Eppenbach Mill to reduce the number and size of the coarse particles.

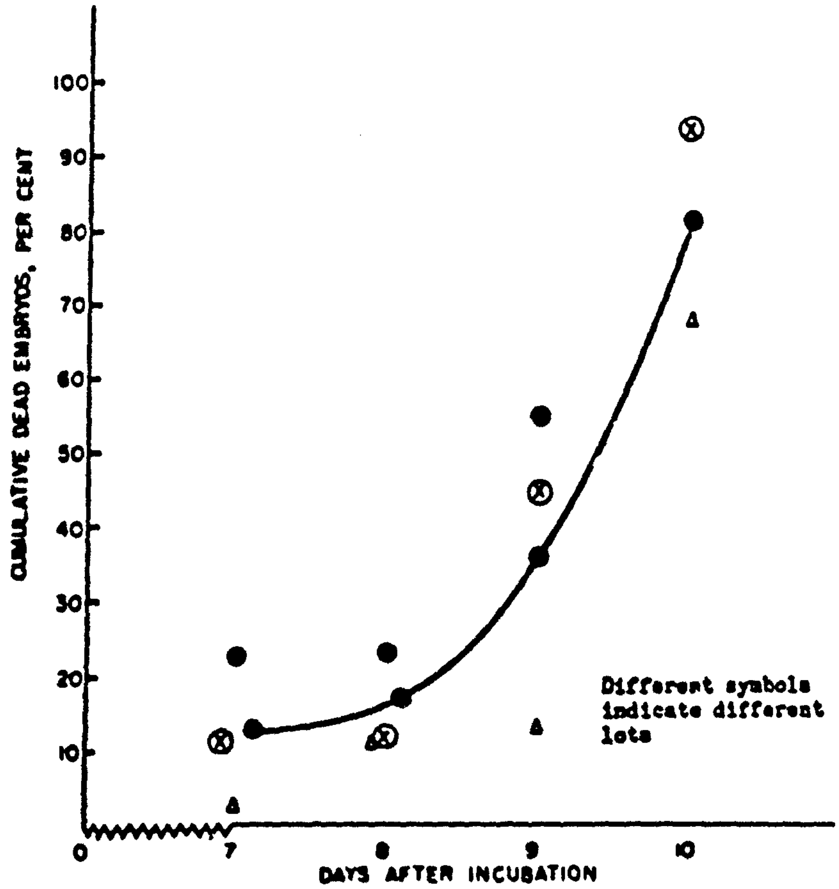

FIGURE 1. (U) DEATH PATTERN OF INFECTED EMBRYONATED EGGS.

(U) It was observed that slurry processed in the Charlotte Mill frequently contains particles that plug the orifice of the Sharples Pressurtite Centrifuge. Although plugging is only momentary, it does disrupt the flow into the centrifuge and makes this operation more difficult to control. It is possible to produce a more refined slurry in the Eppenbach Mill. Considerable debris (shell, bone and feathers) is stopped at the initial cutting blades of the mill and collects in the mill hopper. This debris can be removed either by stopping the operation and increasing the mill setting to pass the debris through the rotor-stator section of the mill to a special container, or by stopping the operation and removing the debris by hand. In either case, the milling operation is delayed. Data indicate that optimum milling can be obtained when slurry is milled first in the Charlotte Mill and then in the Eppenbach Mill.

APPENDIX E

(U) ASSAY VARIABILITY

(U) Assay data for unknown and reference slurries are summarized in Table I. These data were analyzed by F. M. Wadley, Technical Evaluation Division, with particular emphasis given the following relationships: (a) correlation coefficient between unknown and reference assay data, (b) within-day and between-day variability of the reference assay data, (c) within-day and between-day variability of the unknown slurry and (d) difference in assay variability when three and six pigs were inoculated per dilution.

(U) The coefficient of correlation was +0.35 which almost reached the five per cent level of significance with 24 degrees of freedom; of this, about 12 per cent of the variation is accounted for. The variation originates from three main sources:

(a) Inevitable binomial variation of response plus small variations in technique in repeating the same determination; this should not contribute to correlation.

(b) Variation associated with day, affecting both variables alike; this should cause correlation.

(c) Real variation in unknown as compared with an invarying standard; this should not contribute to correlation.

(U) Within-day and between-day variation were studied by analysis of variance and are summarized in Table II.

(U) There was no significant difference in assay variability when three rather than six guinea pigs were used for each inoculating dilution. However, theory and experience show that it is impossible to secure as accurate results with three test animals per dilution as with six.

(U) A slope of guinea pig responses was calculated for the data summarized in Table III. Only five of the twenty series are sufficiently clear-cut for probit analysis; these yield probit slopes of 0.99 to 3.26, averaging 2.17. Since this slope represents selected data, this average is probably too high. These slopes, when averaged with those obtained by Technical Evaluation Division, give a slope of 1.4, which is a better estimate of assay variability.

TABLE I. (U) SUMMARY OF ASSAY DATA OF UNKNOWN AND REFERENCE SLURRIES USED FOR STATISTICAL ANALYSES[a]

Test No.	Unknown Sample[b] Dilution 1	Dilution 2	Reference Sample[b] Dilution 1	Dilution 2
1.	10.50	10.50	10.60	10.50
2.	10.50	10.50		
3.	10.13	9.50	10.00	9.75
4.	10.00	9.73	9.70	10.38
5.	10.50	10.50	10.30	10.50
6.	10.50	10.50		
7.	10.50	10.50	10.25	10.37
8.	10.50	9.90	9.00	9.50
9.	10.50	10.50	10.50	10.50
10.	10.87	11.00		
11.	9.87	10.37		
12.	10.37	11.00	9.50	9.88
13.	10.50	9.87		
14.	10.38	10.40	10.13	10.00
15.	10.00	10.00		
16.	9.73	9.75	10.27	9.88
17.	9.73	10.21[c]	10.33	9.80
18.	11.00	11.00		
19.	10.48	11.00		
20.	9.50	9.60	10.15	9.75
21.	10.19	9.50		
22.	11.00	11.00	11.00	10.80
23.	10.78	11.00		
24.	11.00	10.75		
25.	11.00	10.50	10.27	9.87
26.	10.50	11.00		
27.	10.50	10.50	10.75	
28.	9.25	9.50		
29.	11.00	11.00	10.85	11.00
30.	11.00	10.60		
31.	11.00	11.00	10.33	9.87
32.	10.50	10.17		
33.	11.00	11.00	11.00	11.00
	11.24	10.75		

a. Rickettsial concentration expressed as $\log_{10}$ guinea pig intraperitoneal ID_{50}.
b. Samples for most part consisted of $10^{-9.0}$, $10^{-9.5}$, $10^{-10.0}$ and $10^{-10.50}$ prepared by same technician; three pigs per dilution. In some instances, multiple unknown samples were tested against a single reference sample.
c. Duplicate dilutions prepared by one technician; six pigs per dilution.

TABLE II. (U) ANALYSIS OF VARIANCE IN THE PROCEDURE FOR ASSESSMENT OF COXIELLA BURNETII

Sample Variation	Test Animals[a]	Reference Slurry Degrees of Freedom	Reference Slurry Mean Square	Unknown Slurry Degrees of Freedom	Unknown Slurry Mean Square
Within-Day	3	9	0.0603	9	0.0903
Between-Day	3	8	0.3301[b]	8	0.2965[b]
Within-Day	6	12	0.0751	6	0.0610
Between-Day	6	11	0.6600[b]	5	0.4461[b]

a. Number of guinea pigs inoculated per dilution.
b. Significant past five per cent level.

TABLE III. (U) PER CENT OF ANIMALS INFECTED AFTER CHALLENGE WITH REFERENCE SLURRY

Dilution	Days 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
$10^{-9.0}$										100	100	100			100	100				
$10^{-9.5}$	100	100	100	33	100	100	66	100	100	100	100	100	66	66	100	100	66	66	83	100
$10^{-10.0}$	100	100	66	100	33	100	30	80	100	100	100	100	30	100	66	100	33	83	16	33
$10^{-10.5}$	66	66	100	33	0	100	60	0	100	100	100	100	0	33	33	0	50	16	0	33
$10^{-11.0}$	0	66	0	33	0	100	66	0	100	-	-	-	20	0	-	-	0	0	0	0

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APPENDIX F

(U) CONDITIONS MAINTAINED FOR THE AEROSOL STUDY

(U) The test procedures and conditions maintained in the study of the aerosol properties of whole-egg slurry are tabulated below.

a. Disseminating device - PT-12 Nozzle

b. Impinger Collecting fluid - distilled water

c. Test Chamber - No. 95, Technical Evaluation Division

d. Test Chamber Conditions for each test day:

Date	Relative Humidity, %	Temperature, °F
7 May 58	85	40
8 May 58	85	75
9 May 58	30	75
12 May 58	30	75
13 May 58	85	75
14 May 58	85	40
15 May 58	85	75
16 May 58	85	40
19 May 58	30	75

e. Impinger samples taken at 4, 93, 182, 271, and 360 minutes after creation of the aerosol cloud

f. Impinger fluids diluted in heart infusion broth.

g. Nine animals were used for each time period for each of the five dilutions shown:

Time Period	Dilutions Employed
4 min	10^{-3}, 10^{-4}, 10^{-5} 10^{-6}, 10^{-7}
93 min	10^{-2}, 10^{-3}, 10^{-4} 10^{-5}, 10^{-6}
182 min	10^{-1}, 10^{-2}, 10^{-3} 10^{-4}, 10^{-5}
271 min	10^{0}, 10^{-1}, 10^{-2} 10^{-3}, 10^{-4}
360 min	10^{0}, 10^{-1}, 10^{-2} 10^{-3}, 10^{-4}

h. Control slurry - slurry remaining in PT-12 Nozzle following establishment of aerosol was sent to control laboratory of Egg Process Section and diluted to 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12} in heart infusion broth. Four groups of ten guinea pigs each (one for each dilution) were used. Each guinea pig was inoculated with one milliliter of the pertinent dilution.

i. Holding time of inoculated guinea pigs - 28 days.

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